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(54) Title: MULTIPLY–SUBSTITUTED PROTEASE VARIANTS

(57) Abstract

Novel protease variants derived from the DNA sequences of naturally–occurring or recombinant non–human proteases are disclosed. The variant proteases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding the naturally–occurring or recombinant protease to generate the substitution of a plurality of amino acid residues in the amino acid sequence of a precursor protease. Such variant proteases have properties which are different from those of the precursor protease, such as altered wash performance. The substituted amino acid residue corresponds to position 103 in combination with one or more of the following substitutions at residue positions corresponding to positions 1, 3, 4, 8, 10, 12, 13, 15, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274, and 275 of *Bacillus amyloliquefaciens* subtilisin, wherein when a substitution at a position corresponding to residue position 103 is combined with a substitution at a position corresponding to residue position 76, there is also a substitution at one or more residue positions other than residue positions corresponding to positions 27, 99, 101, 104, 107, 109, 123, 128, 166, 204, 206, 210, 216, 217, 218, 222, 260, 265 or 274 of *Bacillus amyloliquefaciens* subtilisin.

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MULTIPLY-SUBSTITUTED PROTEASE VARIANTS

Related Applications

The present application is a continuation-in-part application of United States Patent Application 08/956,323, filed October 23, 1998, United States Patent Application 08/956,564, filed October 23, 1998, and United States Patent Application 08/956,324 filed October 23, 1998, all of which are hereby incorporated herein in their entirety.

Background of the Invention

Serine proteases are a subgroup of carbonyl hydrolases. They comprise a diverse class of enzymes having a wide range of specificities and biological functions. Stroud, R. Sci. Amer., **131**:74-88. Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: 1) the subtilisins and 2) the mammalian chymotrypsin-related and homologous bacterial serine proteases (e.g., trypsin and *S. gresius* trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis. Kraut, J. (1977), Annu. Rev. Biochem., **46**:331-358. Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families bring together a conserved catalytic triad of amino acids consisting of serine, histidine and aspartate.

Subtilisins are serine proteases (approx. MW 27,500) which are secreted in large amounts from a wide variety of *Bacillus* species and other microorganisms. The protein sequence of subtilisin has been determined from at least nine different species of *Bacillus*. Markland, F.S., et al. (1983), Hoppe-Seyler's Z. Physiol. Chem., **364**:1537-1540. The three-dimensional crystallographic structure of subtilisins from *Bacillus amyloliquefaciens, Bacillus licheniformis* and several natural variants of *B. lentus* have been reported. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972), Biochemistry, **11**:2439-2449) or product complexes (Robertus, J.D., et al. (1976), J. Biol. Chem., **251**:1097-1103) have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical

Biol. Chem., **244**:5333-5338) and extensive site-specific mutagenesis has been carried out (Wells and Estell (1988) TIBS 13:291-297)

Summary of the Invention

It is an object herein to provide protease variants containing a substitution of an amino acid at a residue position corresponding to position 103 of *Bacillus amyloliquefaciens* subtilisin and substituting one or more amino acids at residue positions selected from the group consisting of residue positions corresponding to positions 1, 3, 4, 8, 10, 12, 13, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274 and 275 of *Bacillus amyloliquefaciens* subtilisin; wherein when a substitution at a position corresponding to residue position 103 is combined with a substitution at a position corresponding to residue position 76, there is also a substitution at one or more residue positions other than residue positions corresponding to positions 27, 99, 101, 104, 107, 109, 123, 128, 166, 204, 206, 210, 216, 217, 218, 222, 260, 265, or 274 of *Bacillus amyloliquefaciens* subtilisin.

While any combination of the above listed amino acid substitutions may be employed, the preferred protease variant enzymes useful for the present invention comprise the substitution of amino acid residues in the following combinations of positions. All of the residue positions correspond to positions of *Bacillus amyloliquefaciens* subtilisin:

(1) a protease variant including substitutions of the amino acid residues at position 103 and at one or more of the following positions 236 and 245;

(2) a protease variant including substitutions of the amino acid residues at positions 103 and 236 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 211, 212, 213, 215, 217, 230, 232, 248, 252, 257, 260, 270 and 275;

(3) a protease variant including substitutions of the amino acid residues at positions 103 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 104, 109, 130, 131, 159, 170, 183, 185, 205, 209, 210, 211, 212, 213, 215, 217, 222, 230, 232, 248, 252, 257, 260, 261, 270 and 275; or

(4) a protease variant including substitutions of the amino acid residues at positions 103, 236 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 211, 212, 213, 215, 217, 230, 232, 243, 248, 252, 257, 260, 270 and 275.

More preferred protease variants are substitution sets selected from the group consisting of residue positions corresponding to positions in Table 1 of *Bacillus amyloliquefaciens* subtilisin:

Table 1

76	103	104	222																				
76	98	103	104																				
76	78	103	104																				
76	103	104	107																				
4	76	103	104																				
76	103	104	246																				
76	77	103	104																				
76	103	104	183	218																			
16	76	103	104	248																			
1	76	103	104																				
76	103	104	261																				
76	103	104	160																				
76	103	104	216																				
17	76	103	104																				
37	76	103	104																				
76	77	103	104	174																			
38	76	103	104																				
38	76	103	104	237																			
8	76	103	104																				
76	103	104	183																				

19	76	103	104																				
13	76	103	104																				
19	76	103	104																				
76	103	104	184																				
76	103	104	252																				
76	103	104	259																				
76	103	104	251																				
76	86	103	104																				
72	76	103	104	185																			
76	103	104	237	274																			
76	103	104	160	[illegible]																			
76	103	104	228																				
55	76	103	104	240																			
76	103	104	254																				
76	103	104	204																				
76	103	104	204																				
43	76	103	104																				
76	103	104	159																				
10	76	103	104	177																			
58	76	103	104																				
76	103	104	270																				

76	103	104	185																					
27	76	103	104																					
76	103	104	262																					
76	78	103	104																					
24	76	103	104																					
76	103	104	166	236	251																			
17	76	103	104	237																				
76	103	104	130																					
76	103	104	109																					
76	99	103	104	204																				
76	103	104	181																					
12	76	103	104																					
76	103	104	212	271																				
76	103	104	252	261																				
76	103	104	242																					
76	103	104	271																					
12	76	103	104	242																				
43	76	103	104	116	183																			
76	103	104	258																					
76	103	104	271																					
61	76	103	104																					

38	76	103	104	182	263																			
76	103	104	182	272																				
76	103	104	109	246																				
76	87	103	104	206	249	265																		
76	103	104	137	238	271																			
103	104	228																						
76	103	104	182	198																				
21	76	103	104	182																				
76	103	104	119	137																				
76	103	104	137	248																				
13	76	103	104	206																				
76	103	104	206																					
76	103	104	212	258																				
58	76	103	104	271																				
76	103	104	206	261																				
4	76	103	104	206																				
76	77	103	104	206																				
76	103	104	158																					
76	103	104	206																					
4	76	103	104	159	217	251																		
4	76	103	104	159	217	252																		

76	77	103	104	133	185	251																			
76	103	104	159	206	244																				
4	76	103	104	188																					
4	76	103	104	158																					
76	77	103	104	185																					
76	103	104	206	251																					
48	76	103	104	111	159																				
68	76	103	104	159	236																				
42	76	103	104	159																					
12	62	76	103	104	159																				
42	76	103	104	159																					
76	103	104	146	159																					
76	103	104	159	238																					
76	103	104	159	224																					
76	103	104	212	268	271																				
76	89	103	104																						
76	87	103	104	212	271																				
76	103	104	212	245	271																				
76	103	104	134	141	212	271																			
76	103	104	212	236	243	271																			
76	103	104	109	245																					

76	103	104	109	210																			
20	62	76	103	104																			
68	76	103	104	236																			
68	76	103	104	159	236	271																	
68	76	103	104	159	236	245																	
68	76	103	104	159	217	236	271																
17	68	76	103	104																			
68	76	103	104																				
68	76	103	104	159	236																		
68	75	76	103	104	159	236																	
68	76	76	103	114	121	159	236	245															
12	68	76	103	104	159	236																	
68	76	103	104	159	209	236	253																
68	76	103	104	117	159	184	236																
68	76	103	104	159	236	243																	
68	76	103	104	159	236	245																	
68	76	103	104	142	159																		
68	76	103	104	123	159	236	249																
68	76	103	104	159	236	249																	
76	103	104	222	245																			
12	76	103	104	222	249																		

76	103	104	173	222																			
76	103	104	222	263																			
21	76	103	104	222	237	263																	
76	103	104	109	222																			
76	103	104	109	222	271																		
61	76	103	104	222																			
76	103	104	137	222																			
76	103	104	109	222	248																		
76	103	104	222	249																			
68	76	103	104	159	236	245	261																
68	76	103	104	141	159	236	245	255															
68	76	103	104	159	236	245	247																
68	76	103	104	159	174	204	236	245															
68	76	103	104	159	204	236	245																
68	76	103	104	133	159	218	236	245															
68	76	103	104	159	232	236	245																
68	76	103	104	159	194	203	236	245															
12	76	103	104	222	245																		
76	103	104	232	245																			
24	68	76	103	104	159	232	236	245															
68	103	104	159	232	236	245	252																

68	76	103	104	159	213	232	236	245	260			
12	76	103	104	222	244	245						
12	76	103	222	210	245							
12	76	103	104	130	222	245						
22	68	76	103	104								
68	76	103	104	184								
68	103	104	159	232	236	245	248	252				
68	103	104	159	232	236	245						
68	103	104	140	159	232	236	245	252				
43	68	103	104	159	232	236	245	252				
43	68	103	104	159	232	236	245					
43	68	103	104	159	232	236	245	252				
68	87	103	104	159	232	236	245	252	275			
12	76	103	104	130	222	245	248	262				
12	76	103	104	130	215	222	245					
12	76	103	104	130	222	227	245	262				
12	76	103	104	130	222	245	261					
76	103	104	130	222	245							
12	76	103	104	130	218	222	245	262	269			
12	57	76	103	104	130	222	245	251				
12	76	103	104	130	170	185	222	243	245			

12	76	103	104	130	222	245	268																	
12	76	103	104	130	222	210	245																	
68	103	104	159	232	236	245	257																	
68	103	104	116	159	232	236	245																	
68	103	104	159	232	236	245	248																	
10	68	103	104	159	232	236	245																	
68	103	104	159	203	232	236	245																	
68	103	104	159	232	236	237	245																	
68	76	79	103	104	159	232	236	245																
68	103	104	159	183	232	236	245																	
68	103	104	159	174	206	232	236	245																
68	103	104	159	188	232	236	245																	
68	103	104	159	230	232	236	245																	
68	98	103	104	159	232	236	245																	
68	103	104	159	215	232	236	245																	
68	103	104	159	232	236	245	248																	
68	76	103	104	159	232	236	245																	
68	76	103	104	159	210	232	236	245																
68	76	103	104	159	232	236	245	257																
76	103	104	232	236	245	257																		
68	103	104	159	232	236	245	257	275																

76	103	104	257	275																				
68	103	104	159	224	232	236	245	257																
76	103	104	159	232	236	245	257																	
68	76	103	104	159	209	232	236	245																
68	76	103	104	159	211	232	236	245																
12	68	76	103	104	159	214	232	236	245															
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12	68	76	103	104	159	232	236	245																
20	68	76	103	104	159	232	236	245	259															
68	87	76	103	104	159	232	236	245	260															
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76	103	104	232	236	242	245																		
68	76	103	104	159	210	232	236	245																
12	48	68	76	103	104	159	232	236	245															
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68	76	103	104	159	206	232	236	245																

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43	68	103	104	159	232	236	245	248	252					
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68	103	104	99	159	184	232	236	245	248	252				
103	104	159	232	236	245	248	252							
68	103	104	159	209	232	236	245	248	252					
68	103	104	109	159	232	236	245	248	252					
20	68	103	104	159	232	236	245	248	252					
68	103	104	159	209	232	236	245	248	252					
68	103	104	159	232	236	245	248	252	261					
68	103	104	159	185	232	236	245	248	252					
68	103	104	159	210	232	236	245	248	252					
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68	103	104	159	212	232	236	245	248	252					
68	103	104	159	213	232	236	245	248	252					
68	103	104	213	232	236	245	248	252						
68	103	104	159	215	232	236	245	248	252					
68	103	104	159	216	232	236	245	248	252					
20	68	103	104	159	232	236	245	248	252					

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68	103	104	159	206	232	236	245	248	252			
68	103	104	159	232	236	245	248	252				
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68	103	104	159	232	236	245	248	252	255			
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8	68	103	104	159	232	236	245	248	252	269		
68	103	104	116	159	232	236	245	248	252	260		
68	103	104	159	232	236	245	248	252	261			
68	103	104	159	232	236	245	248	252	261			
68	76	103	104	159	232	236	245	248	252			
68	103	104	232	236	245	248	252					
103	104	159	232	236	245	248	252					
68	103	104	159	232	236	245	248	252				
18	68	103	104	159	232	236	245	248	252			
68	103	104	159	232	236	245	248	252				
68	76	101	103	104	159	213	218	232	236	245	260	

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33	68	76	103	104	159	232	236	245	248	252			
68	76	89	103	104	159	210	213	232	236	245	260		
61	68	76	103	104	159	232	236	245	248	252			
103	104	159	205	210	232	236	245						
61	68	103	104	130	159	232	236	245	248	252			
61	68	103	104	133	137	159	232	236	245	248	252		
61	103	104	133	159	232	236	245	248	252				
68	103	104	159	232	236	245	248	252					
68	103	104	159	218	232	236	245	248	252				
61	68	103	104	159	160	232	236	245	248	252			
3	61	68	76	103	104	232	236	245	248	252			
61	68	103	104	159	167	232	236	245	248	252			
97	103	104	159	232	236	245	248	252					
98	103	104	159	232	236	245	248	252					
99	103	104	159	232	236	245	248	252					
101	103	104	159	232	236	245	248	252					
102	103	104	159	232	236	245	248	252					
103	104	106	159	232	236	245	248	252					
103	104	109	159	232	236	245	248	252					
103	104	159	232	236	245	248	252	261					

62	103	104	159	232	236	245	248	252						
103	104	159	184	232	236	245	248	252						
103	104	159	166	232	236	245	248	252						
103	104	159	217	232	236	245	248	252						
20	62	103	104	159	213	232	236	245	248	252				
62	103	104	159	213	232	236	245	248	252					
103	104	159	206	217	232	236	245	248	252					
62	103	104	159	206	232	236	245	248	252					
103	104	130	159	232	236	245	248	252						
103	104	131	159	232	236	245	248	252						
27	103	104	159	232	236	245	248	252						
38	103	104	159	232	236	245	248	252						
38	76	103	104	159	213	232	236	245	260					
68	76	103	104	159	213	232	236	245	260	271				
68	76	103	104	159	209	213	232	236	245	260				
68	76	103	104	159	210	213	232	236	245	260				
68	76	103	104	159	205	213	232	236	245	260				
68	76	103	104	159	210	232	236	245	260					
68	103	104	159	213	232	236	245	260						
76	103	104	159	213	232	236	245	260						
68	103	104	159	209	232	236	245							

68	103	104	159	210	232	236	245																
68	103	104	159	230	232	236	245																
68	103	104	159	126	232	236	245																
68	103	104	159	205	232	236	245																
68	103	104	159	210	232	236	245																
103	104	159	230	236	245																		
68	103	104	159	232	236	245	260																
103	104	159	232	236	245																		
68	103	104	159	174	232	236	245	257															
68	103	104	159	194	232	236	245	257															
68	103	104	159	209	232	236	245	257															
103	104	159	232	236	245	257																	
68	76	103	104	159	213	232	236	245	260	261													
68	103	104	159	232	236	245	257	261															
103	104	159	213	232	236	245	260																
103	104	159	210	232	236	245	248	252															
103	104	159	209	232	236	245	257																
68	76	103	104	159	210	213	232	236	245	260													
12	103	104	159	209	213	232	236	245	260														
103	104	209	232	236	245	257																	
103	104	159	205	210	213	232	236	245	260														

103	104	159	205	209	232	236	245	260					
68	103	104	159	205	209	210	232	236	245				
103	104	159	205	209	210	232	236	245	257				
103	104	159	205	209	232	236	245	257					
68	103	104	159	205	209	210	232	236	245	260			
103	104	159	205	209	210	232	236	245					
103	104	159	209	210	232	236	245						
103	104	159	205	210	232	236	245						
68	103	104	128	159	232	236	245						
48	103	104	159	230	236	245							
48	68	103	104	159	209	232	236	245					
48	68	103	104	159	232	236	245	248	252				
48	68	103	104	159	232	236	245	257	261				
102	103	104	159	212	232	236	245	248	252				
12	102	103	104	159	212	232	236	245	248	252			
101	102	103	104	159	212	232	236	245	248	252			
98	102	103	104	159	212	232	236	245	248	252			
102	103	104	159	213	232	236	245	248	252				
103	104	131	159	232	236	245	248	252					
103	104	159	184	232	236	245	248	252					
103	104	159	232	236	244	245	248	252					

62	103	104	159	213	232	236	245	248	252	256		
12	62	103	104	159	213	232	236	245	248	252		
101	103	104	159	185	232	236	245	248	252			
101	103	104	159	206	232	236	245	248	252			
101	103	104	159	213	232	236	245	248	252			
98	102	103	104	159	232	236	245	248	252			
101	102	103	104	159	232	236	245	248	252			
98	102	103	104	159	212	232	236	245	248	252		
98	102	103	104	159	212	232	236	248	252			
62	103	104	109	159	213	232	236	245	248	252		
62	103	104	159	212	213	232	236	245	248	252		
62	101	103	104	159	212	213	232	236	245	248	252	
103	104	159	232	245	248	252						
103	104	159	230	245								
62	103	104	130	159	213	232	236	245	248	252		
101	103	104	130	159	232	236	245	248	252			
101	103	104	128	159	232	236	245	248	252			
62	101	103	104	159	213	232	236	245	248	252		
62	103	104	128	159	213	232	236	245	248	252		
62	103	104	128	159	213	232	236	245	248	252		
101	103	104	159	232	236	245	248	252	260			

101	103	104	131	159	232	236	245	248	252					
98	101	103	104	159	232	236	245	248	252					
99	101	103	104	159	232	236	245	248	252					
101	103	104	159	212	232	236	245	248	252					
101	103	104	159	209	232	236	245	248	252					
101	103	104	159	210	232	236	245	248	252					
101	103	104	159	205	232	236	245	248	252					
101	103	104	159	230	236	245								
101	103	104	159	194	232	236	245	248	252					
76	101	103	104	159	194	232	236	245	248	252				
101	103	104	159	230	232	236	245	248	252					
62	103	104	159	185	206	213	232	236	245	248	252	271		

Most preferred protease variants are those shown in Table 3.

It is a further object to provide DNA sequences encoding such protease variants, as well as expression vectors containing such variant DNA sequences.

Still further, another object of the invention is to provide host cells transformed with such vectors, as well as host cells which are capable of expressing such DNA to produce protease variants either intracellularly or extracellularly.

There is further provided a cleaning composition comprising a protease variant of the present invention.

Additionally, there is provided an animal feed comprising a protease variant of the present invention.

Also provided is a composition for the treatment of a textile comprising a protease variant of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene.

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN'). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis*. The third line depicts the amino acid sequence of subtilisin from *B. licheniformis*. The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Detailed Description of the Invention

Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include α-aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

The present invention includes protease enzymes which are non-naturally occurring carbonyl hydrolase variants (protease variants) having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. Specifically, such protease variants have an amino acid sequence not found in nature, which is derived by substitution of a plurality of amino acid residues of a precursor protease with different amino acids. The precursor protease may be a naturally-occurring protease or a recombinant protease.

The protease variants useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one - and three-letter codes. Such codes are identified in Dale, M.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

The protease variants useful herein are preferably derived from a *Bacillus* subtilisin. More preferably, the protease variants are derived from *Bacillus lentus* subtilisin and/or subtilisin 309.

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1.

"Recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent RE 34,606, US Patent 5,204,015 and US Patent 5,185,258, U.S. Patent 5,700,676, U.S. Patent 5,801,038, and U.S. Patent 5,763,257.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

A "protease variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor protease". The precursor proteases include naturally-occurring proteases and recombinant proteases. The amino acid sequence of the protease variant is "derived" from the precursor protease amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor protease rather than manipulation of the precursor protease enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific substitutions corresponding to position 103 in combination with one or more of the following substitutions corresponding to positions 1, 3, 4, 8, 10, 12, 13, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274 and 275 of *Bacillus amyloliquefaciens* subtilisin are identified herein.

Preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 1. More preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 3.

Further preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 2.

Table 2

76	103	104	222	245																	
76	103	104	222	249																	
68	103	104	159	232	236	245	252														
68	76	103	104	159	213	232	236	245	260												
22	68	76	103	104																	
68	103	104	159	232	236	245	248	252													
68	103	104	159	232	236	245															
68	103	104	140	159	232	236	245	252													
43	68	103	104	159	232	236	245	252													
43	68	103	104	159	232	236	245														
12	76	103	104	130	222	245	261														
76	103	104	130	222	245																
68	103	104	159	232	236	245	257														
68	76	103	104	159	210	232	236	245													
68	103	104	159	224	232	236	245	257													
76	103	104	159	232	236	245	257														
68	76	103	104	159	211	232	236	245													
12	68	76	103	104	159	214	232	236	245												
68	76	103	104	159	215	232	236	245													

12	68	76	103	104	159	232	236	245		259													
20	68	76	103	104	159	232	236	245	259														
68	76	87	103	104	159	232	236	245	260														
68	76	103	104	159	232	236	245	261															
12	48	68	76	103	104	159	232	236	245														
76	103	104	159	192	232	236	245																
76	103	104	147	159	232	236	245	248	251														
12	68	76	103	104	159	232	236	245	272														
68	76	103	104	159	183	206	232	236	245														
68	76	103	104	159	232	236	245	256															
68	76	103	104	159	206	232	236	245															
27	68	76	103	104	159	232	236	245															
68	103	104	159	212	232	236	245	248	252														
103	104	159	232	236	245	248	252																
68	103	104	159	209	232	236	245	248	252														
68	103	104	109	159	232	236	245	248	252														
20	68	103	104	159	232	236	245	248	252														
68	103	104	159	209	232	236	245	248	252														
68	103	104	159	210	232	236	245	248	252														
68	103	104	159	212	232	236	245	248	252														

68	103	104	159	213	232	236	245	248	252				
68	103	104	213	232	236	245	248	252					
68	103	104	159	215	232	236	245	248	252				
68	103	104	159	216	232	236	245	248	252				
20	68	103	104	159	232	236	245	248	252				
68	103	104	159	232	236	245	248	252	255				
68	103	104	159	232	236	245	248	252	256				
68	103	104	159	232	236	245	248	252	260				
68	103	104	159	232	236	236	245	248	252				
68	103	104	159	228	232	236	245	248	252				
68	76	89	103	104	159	210	213	232	236	245	260		
68	103	104	159	218	232	236	245	248	252				

These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions are made at the equivalent amino acid residue positions in *B. lentus* corresponding to those listed above.

A residue (amino acid) position of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained. Siezen et al. (1991) Protein Eng. **4(7)**:719-737 shows the alignment of a large number of serine proteases. Siezen et al. refer to the grouping as subtilases or subtilisin-like serine proteases.

For example, in Fig. 3, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protease whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R\,factor = \frac{\Sigma_h |Fo(h)| - |Fc(h)|}{\Sigma_h |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the

main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the substitution of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such substitutions should not result in a naturally-occurring sequence. The protease variants of the present invention include the mature forms of protease variants, as well as the pro- and prepro-forms of such protease variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the protease variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protease which when removed results in the appearance of the "mature" form of the protease. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protease variants is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other protease prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protease or to the N-terminal portion of a proprotease which may participate in the secretion of the mature or pro forms of the protease. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the protease gene which participate in the effectuation of the secretion of protease under native conditions. The present invention utilizes such sequences to effect the secretion of the protease variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a protease variant consists of the mature form of the protease having a prosequence operably linked to the amino terminus of the protease and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent RE 34,606 to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protease is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protease include *Bacillus subtilis* I168 (also described in US Patent RE 34,606 and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis, B. lentus*, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the protease variants or expressing the desired protease variant. In the case of vectors which encode the pre- or prepro-form of the protease variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked, " when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence,

participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor protease may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protease of interest, preparing genomic libraries from organisms expressing the protease, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned protease is then used to transform a host cell in order to express the protease. The protease gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protease gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protease gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the protease gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

The gene can be a natural *B. lentus* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protease may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor protease is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protease. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protease gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protease. Such modifications include the production of recombinant proteases as disclosed in US Patent RE 34,606 and EPO Publication No. 0 251 446 and the production of protease variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protease variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protease is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protease gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protease gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as K_m, k_{cat}, k_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, in the preparation of peptides or for hydrolytic processes such as laundry uses.

In one aspect of the invention, the objective is to secure a variant protease having altered, preferably improved wash performance as compared to a precursor protease in at least one detergent formulation and or under at least one set of wash conditions.

There is a variety of wash conditions including varying detergent formulations, wash water volume, wash water temperature and length of wash time that a protease variant might be exposed to. For example, detergent formulations used in different areas have different concentrations of their relevant components present in the wash water. For example, a European detergent typically has about 4500-5000 ppm of detergent components in the wash water while a Japanese detergent typically has approximately 667 ppm of detergent components in the wash water. In North America, particularly the United States, a detergent typically has about 975 ppm of detergent components present in the wash water.

A low detergent concentration system includes detergents where less than about 800 ppm of detergent components are present in the wash water. Japanese detergents are typically considered low detergent concentration system as they have approximately 667 ppm of detergent components present in the wash water.

A medium detergent concentration includes detergents where between about 800 ppm and about 2000ppm of detergent components are present in the wash water. North American detergents are generally considered to be medium detergent concentration systems as they have approximately 975 ppm of detergent components present in the wash water. Brazil typically has approximately 1500 ppm of detergent components present in the wash water.

A high detergent concentration system includes detergents where greater than about 2000 ppm of detergent components are present in the wash water. European detergents are generally considered to be high detergent concentration systems as they have approximately 4500-5000 ppm of detergent components in the wash water.

Latin American detergents are generally high suds phosphate builder detergents and the range of detergents used in Latin America can fall in both the medium and high detergent concentrations as they range from 1500 ppm to 6000 ppm of detergent components in the wash water. As mentioned above, Brazil typically has approximately 1500 ppm of detergent components present in the wash water. However, other high suds phosphate builder detergent geographies, not limited to other Latin American countries, may have high detergent concentration systems up to about 6000 ppm of detergent components present in the wash water.

In light of the foregoing, it is evident that concentrations of detergent compositions in typical wash solutions throughout the world varies from less than about 800 ppm of detergent composition ("low detergent concentration geographies"), for example about 667 ppm in Japan, to between about 800 ppm to about 2000 ppm ("medium detergent concentration geographies"), for example about 975 ppm in U.S. and about 1500 ppm in Brazil, to greater than about 2000 ppm ("high detergent concentration geographies"), for example about 4500 ppm to about 5000 ppm in Europe and about 6000 ppm in high suds phosphate builder geographies.

The concentrations of the typical wash solutions are determined empirically. For example, in the U.S., a typical washing machine holds a volume of about 64.4 L of wash solution. Accordingly, in order to obtain a concentration of about 975 ppm of detergent within the wash solution about 62.79 g of detergent composition must be added to the 64.4 L of wash solution. This amount is the typical amount measured into the wash water by the consumer using the measuring cup provided with the detergent.

As a further example, different geographies use different wash temperatures. The temperature of the wash water in Japan is typically less than that used in Europe.

Accordingly one aspect of the present invention includes a protease variant that shows improved wash performance in at least one set of wash conditions.

In another aspect of the invention, it has been determined that substitutions at a position corresponding to 103 in combination with one or more substitutions selected from the group consisting of positions corresponding 1, 3, 4, 8, 10, 12, 13, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256,

257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274 and 275 of *Bacillus amyloliquefaciens* subtilisin are important in improving the wash performance of the enzyme.

These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protease.

Based on the screening results obtained with the variant proteases, the noted mutations in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protease variants of the invention are useful in formulating various detergent compositions or personal care formulations such as shampoos or lotions. A number of known compounds are suitable surfactants useful in compositions comprising the protease mutants of the invention. These include nonionic, anionic, cationic, or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protease variants of the present invention may be used for any purpose that native or wild-type proteases are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about 0.01 to about 5% (preferably 0.1% to 0.5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH

is within the above range, and the temperature is below the described protease's denaturing temperature. In addition, proteases of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The present invention also relates to cleaning compositions containing the protease variants of the invention. The cleaning compositions may additionally contain additives which are commonly used in cleaning compositions. These can be selected from, but not limited to, bleaches, surfactants, builders, enzymes and bleach catalysts. It would be readily apparent to one of ordinary skill in the art what additives are suitable for inclusion into the compositions. The list provided herein is by no means exhaustive and should be only taken as examples of suitable additives. It will also be readily apparent to one of ordinary skill in the art to only use those additives which are compatible with the enzymes and other components in the composition, for example, surfactant.

When present, the amount of additive present in the cleaning composition is from about 0.01% to about 99.9%, preferably about 1% to about 95%, more preferably about 1% to about 80%.

The variant proteases of the present invention can be included in animal feed such as part of animal feed additives as described in, for example, US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteases of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

All publications and patents referenced herein are hereby incorporated by reference in their entirety.

Example 1

A large number of protease variants were produced and purified using methods well known in the art. All mutations were made in *Bacillus lentus* GG36 subtilisin. The variants are shown in Table 3.

Table 3

N76D	S103A	V104I	M222S																
N76D	A98E	S103A	V104I																
N76D	S78T	S103A	V104I																
N76D	S103A	V104I	I107V																
V4E	N76D	S103A	V104I																
N76D	S103A	V104I	I246V																
N76D	N77D	S103A	V104I																
N76D	S103A	V104I	N183D	N218I															
A16T	N76D	S103A	V104I	N248D															
A1E	N76D	S103A	V104I																
N76D	S103A	V104I	N261D																
N76D	S103A	V104I	S160T																
N76D	S103A	V104I	S216C																
H17Q	N76D	S103A	V104I																
S37T	N76D	S103A	V104I																
N76D	N77D	S103A	V104I	A174V															
T38S	N76D	S103A	V104I																

T38S	N76D	S103A	V104I	K237Q								
I8V	N76D	S103A	V104I									
N76D	S103A	V104I	N183D									
R19L	N76D	S103A	V104I									
A13V	N76D	S103A	V104I									
R19C	N76D	S103A	V104I									
N76D	S103A	V104I	N184D									
N76D	S103A	V104I	N252D									
N76D	S103A	V104I	S259C									
N76D	S103A	V104I	K251T									
N76D	P86S	S103A	V104I									
I72V	N76D	S103A	V104I	N185D								
N76D	S103A	V104I	K237E	T274A								
N76D	S103A	V104I	S160L									
N76D	S103A	V104I	A228V									
P55S	N76D	S103A	V104I	S240T								
N76D	S103A	V104I	A254T									
N76D	S103A	I104N	N204T									
N76D	S103A	V104I	N204D									

N43S	N76D	S103A	V104I																		
N76D	S103A	V104I	G159D																		
R10H	N76D	S103A	V104I	V177A																	
T58S	N76D	S103A	V104I																		
N76D	S103A	V104I	A270V																		
N76D	S103A	V104I	N185D																		
K27N	N76D	S103A	V104I																		
N76D	S103A	V104I	L262M																		
N76D	S78P	S103A	V104I																		
S24P	N76D	S103A	V104I																		
N76D	S103A	V104I	S166G	Q236R	K251R																
H17L	N76D	S103A	V104I	K237E																	
N76D	S103A	V104I	S130L																		
N76D	S103A	V104I	Q109R																		
N76D	S99R	S103A	V104I	N204T																	
N76D	S103A	V104I	D181N																		
Q12R	N76D	S103A	V104I																		
N76D	S103A	V104I	S212P	E271V																	
N76D	S103A	V104I	N252K	N261Y																	

N76D	S103A	V104I	S242T																				
N76D	S103A	V104I	E271Q																				
Q12R	N76D	S103A	V104I	S242T																			
N43S	N76D	S103A	V104I	N116K	N183I																		
N76D	S103A	V104I	G258R																				
N76D	S103A	V104I	E271G																				
G61R	N76D	S103A	V104I																				
T38S	N76D	S103A	V104I	Q182R	Y263H																		
N76D	S103A	V104I	Q182R	A272S																			
N76D	S103A	V104I	Q109R	I246V																			
N76D	S87G	S103A	V104I	Q206R	H249Q	S265G																	
N76D	S103A	V104I	Q137R	N238Y	E271V																		
S103A	V104I	A228T																					
N76D	S103A	V104I	Q182R	I198V																			
L21M	N76D	S103A	V104I	Q182R																			
N76D	S103A	V104I	M119I	Q137R																			
N76D	S103A	V104I	Q137R	N248S																			
A13T	N76D	S103A	V104I	Q206R																			
N76D	S103A	V104I	Q206R																				

N76D	S103A	V104I	S212P	G258R																				
T58S	N76D	S103A	V104I	E271G																				
N76D	S103A	V104I	Q206E	N261D																				
V4E	N76D	S103A	V104I	Q206E																				
N76D	N77D	S103A	V104I	Q206E																				
N76D	S103A	V104I	A158E																					
N76D	S103A	V104I	Q206E																					
V4E	N76D	S103A	V104I	G159D	L217E	K251Q																		
V4E	N76D	S103A	V104I	G159D	L217E	N252D																		
N76D	N77D	S103A	V104I	A133T	N185D	K251T																		
N76D	S103A	V104I	G159D	Q206E	V244A																			
V4E	N76D	S103A	V104I	S188E																				
V4E	N76D	S103A	V104I	A158E																				
N76D	N77D	S103A	V104I	N185D																				
N76D	S103A	V104I	Q206E	K251T																				
A48T	N76D	S103A	V104I	L111M	G159D																			
V68A	N76D	S103A	V104I	G159D	Q236H																			
L42V	N76D	S103A	V104I	G159D																				
Q12H	N62H	N76D	S103A	V104I	G159D																			

L42I	N76D	S103A	V104I	G159D																			
N76D	S103A	V104I	G146S	G159D																			
N76D	S103A	V104I	G159D	N238S																			
N76D	S103A	V104I	G159D	T224A																			
N76D	S103A	V104I	S212P	V268F	E271V																		
N76D	E89A	S103A	V104I																				
N76D	S87R	S103A	V104I	S212P	E271V																		
N76D	S103A	V104I	S212P	Q245L	E271V																		
N76D	S103A	V104I	T134S	S141N	S212P	E271V																	
N76D	S103A	V104I	S212P	Q236L	N243S	E271V																	
N76D	S103A	V104I	Q109R	Q245R																			
N76D	S103A	V104I	Q109R	P210L																			
G20V	N62S	N76D	S103A	V104I																			
V68A	N76D	S103A	V104I	Q236H																			
V68A	N76D	S103A	V104I	G159D	Q236H	E271V																	
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R																	
V68A	N76D	S103A	V104I	G159D	L217I	Q236H	E271V																
H17Q	V68A	N76D	S103A	V104I																			
V68A	N76D	S103A	V104I																				

V68A	N76D	S103A	V104I	G159D	Q236R																		
V68A	L75R	N76D	S103A	V104I	G159D	Q236H																	
V68A	N76D	N76D	S103A	A114V	V121I	G159D	Q236H	Q245R															
Q12R	V68A	N76D	S103A	V104I	G159D	Q236H																	
V68A	N76D	S103A	V104I	G159D	Y209S	Q236H	T253K																
V68A	N76D	S103A	V104I	N117K	G159D	N184S	Q236H																
V68A	N76D	S103A	V104I	G159D	Q236H	N243I																	
V68A	N76D	S103A	V104I	G159D	Q236H	Q245L																	
V68A	N76D	S103A	V104I	A142V	G159D																		
V68A	N76D	S103A	V104I	N123S	G159D	Q236H	H249Y																
V68A	N76D	S103A	V104I	G159D	Q236H	H249Q																	
N76D	S103A	V104I	M222S	Q245R																			
N76D	S103A	V104I	Q12R	M222S	H249R																		
N76D	S103A	V104I	N173R	M222S																			
N76D	S103A	V104I	M222S	Y263F																			
L21M	N76D	S103A	V104I	M222S	K237R	Y263F																	
N76D	S103A	V104I	Q109R	M222S																			
N76D	S103A	V104I	Q109R	M222S	E271D																		
G61R	N76D	S103A	V104I	M222S																			

N76D	S103A	V104I	Q137R	M222S								
N76D	S103A	V104I	Q109R	M222S	N248S							
N76D	S103A	V104I	M222S	H249R								
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R	N261D					
V68A	N76D	S103A	V104I	S141N	G159D	Q236H	Q245R	T255S				
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R	R247H					
V68A	N76D	S103A	V104I	G159D	A174V	N204D	Q236H	Q245R				
V68A	N76D	S103A	V104I	G159D	N204D	Q236H	Q245R					
V68A	N76D	S103A	V104I	A133V	G159D	N218D	Q236H	Q245R				
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R					
V68A	N76D	S103A	V104I	G159D	A194I	V203A	Q236H	Q245R				
Q12R	N76D	S103A	V104I	M222S	Q245R							
N76D	S103A	V104I	A232V	Q245R								
S24T	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R				
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K					
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A			
Q12R	N76D	S103A	I104T	M222S	V244I	Q245R						
Q12R	N76D	S103A	M222S	P210T	Q245R							
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R						

T22K	V68A	N76D	S103A	V104I										
V68A	N76D	S103A	N184D											
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K						
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R								
V68A	S103A	V104I	N140D	G159D	A232V	Q236H	Q245R	N252K						
N43S	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K						
N43K	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R							
N43D	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K						
V68A	S87G	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K	R275S					
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R	N248S	L262M						
Q12R	N76D	S103A	I104T	S130T	A215V	M222S	Q245R							
Q12R	N76D	S103A	I104T	S130T	M222S	V227A	Q245R	L262S						
Q12R	N76D	S103A	I104T	S130T	A215T	M222S	Q245R							
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R	N261D							
N76D	S103A	I104T	S130T	M222S	Q245R									
Q12R	N76D	S103A	I104T	S130T	N218D	M222S	Q245R	L262S	N269D					
Q12R	S57P	N76D	S103A	I104T	S130T	M222S	Q245R	K251Q						
Q12R	N76D	S103A	I104T	S130T	R170S	N185D	M222S	N243D	Q245R					
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R	V268A							

Q12R	N76D	S103A	I104T	S130T	M222S	P210S	Q245R					
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V					
V68A	S103A	V104I	N116D	G159D	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D					
R10C	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	V203E	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A232V	Q236H	K237E	Q245R					
V68A	N76D	I79N	S103A	V104I	G159D	A232V	Q236H	Q245R				
V68A	S103A	V104I	G159D	N183D	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A174V	Q206L	A232V	Q236H	Q245R				
V68A	S103A	V104I	G159D	S188C	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A230T	A232V	Q236H	Q245R					
V68A	A98T	S103A	V104I	G159D	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A215T	A232V	Q236H	Q245R					
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248S					
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R					
V68A	N76D	S103A	V104I	G159D	P210R	A232V	Q236H	Q245R				
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V				
N76D	S103A	V104I	A232V	Q236H	Q245R	L257V						

V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V	R275H				
N76D	S103A	V104I	L257V	R275H								
V68A	S103A	V104I	G159D	T224A	A232V	Q236H	Q245R	L257V				
N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V					
V68A	N76D	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R				
V68A	N76D	S103A	V104I	G159D	G211R	A232V	Q236H	Q245R				
V68A	N76D	S103A	V104I	G159D	G211V	A232V	Q236H	Q245R				
Q12R	V68A	N76D	S103A	V104I	G159D	Y214L	A232V	Q236H	Q245R			
V68A	N76D	S103A	V104I	G159D	A215R	A232V	Q236H	Q245R				
Q12R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R				
G20R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	S259G			
V68A	S87R	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	T260V			
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N261G				
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N261W				
N76D	S103A	V104I	A232V	Q236H	S242P	Q245R						
V68A	N76D	S103A	V104I	G159D	P210L	A232V	Q236H	Q245R				
Q12R	A48V	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R			
N76D	S103A	V104I	A232V	Q236H	Q245R							
N76D	S103A	V104I	G159D	Y192F	A232V	Q236H	Q245R					

N76D	S103A	V104I	V147I	G159D	A232V	Q236H	Q245R	N248S	K251R			
Q12R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	A272S			
V68A	N76D	S103A	V104I	G159D	N183K	Q206L	A232V	Q236H	Q245R			
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	S256R				
V68A	N76D	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R				
K27R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R				
V68A	N76D	S103A	V104I	N116T	G159D	R170S	N185S	A232V	Q236H	Q245R		
G61E	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
N43D	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	S212P	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	S99N	G159D	N184D	A232V	Q236H	Q245R	N248D	N252K		
S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					
V68A	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	Q109R	G159D	A232V	Q236H	Q245R	N248D	N252K			
G20R	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	Y209F	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261D			
V68A	S103A	V104I	G159D	N185D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	P210R	A232V	Q236H	Q245R	N248D	N252K			

V68A	S103A	V104I	G159D	P210T	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	P210S	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	N185D	P210L	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	P210L	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S212A	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S212E	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	T213E	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	T213S	A232V	Q236H	Q245R	N248D	N252K					
V68A	A103V	V104I	G159D	T213E	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	T213G	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	A215V	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	A215R	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S216T	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S216V	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	S216C	A232V	Q236H	Q245R	N248D	N252K				
G20A	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	N173D	A232V	Q236H	Q245R	N248D	N252K				

V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	K251V	N252K			
V68A	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252F				
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252L				
P55S	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252F			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	T255V			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256N			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256E			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256R			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	T260R			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	L257R			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	G258D			
I8V	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N269D		
V68A	S103A	V104I	N116S	G159D	A232V	Q236H	Q245R	N248D	N252K	T260E		
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261R			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261D			
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	A232V	Q236H	Q245R	N248D	N252K					
S103A	V104I	G159D	A232S	Q236H	Q245R	N248D	N252K					

V68A	S103A	V104I	G159D	A232V	Q236R	Q245R	N248D	N252K				
N18S	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245V	N248D	N252K				
V68A	N76D	S101T	S103A	V104I	G159D	T213R	N218S	A232V	Q236H	Q245R	T260A	
V68A	S103A	V104I	G159D	A228V	A232V	Q236H	Q245R	N248D	N252K			
T33S	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		
V68A	N76D	E89D	S103A	V104I	G159D	P210L	T213R	A232V	Q236H	Q245R	T260A	
G61E	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		
S103A	V104I	G159D	V205I	P210I	A232V	Q236H	Q245R					
G61E	V68A	S103A	V104I	S130A	G159D	A232V	Q236H	Q245R	N248D	N252K		
G61E	V68A	S103A	V104I	A133S	Q137R	G159D	A232V	Q236H	Q245R	N248D	N252K	
G61E	S103A	V104I	A133V	G159D	A232V	Q236H	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248G	N252K				
V68A	S103A	V104I	G159D	N218S	A232V	Q236H	Q245R	N248D	N252K			
G61E	V68A	S103A	V104I	G159D	S160V	A232V	Q236H	Q245R	N248D	N252K		
S3L	G61E	V68A	N76D	S103A	V104I	A232V	Q236H	Q245R	N248D	N252K		
G61E	V68A	S103A	V104I	G159D	S167F	A232V	Q236H	Q245R	N248D	N252K		
G97E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				
A98D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				

S99E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	S106E	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	Q109E	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261R			
S103A	V104I	Q109R	G159D	A232V	Q236H	Q245R	N248D	N252K			
N62D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	N184D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	S166D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	L217E	A232V	Q236H	Q245R	N248D	N252K			
G20R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K	
N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
S103A	V104I	G159D	Q206R	L217E	A232V	Q236H	Q245R	N248D	N252K		
N62D	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R	N248D	N252K		
S103A	V104I	S130G	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	P131V	G159D	A232V	Q236H	Q245R	N248D	N252K			
K27N	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			

T38G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					
T38A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A				
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A	E271G			
V68A	N76D	S103A	V104I	G159D	Y209W	T213R	A232V	Q236H	Q245R	T260A			
V68A	N76D	S103A	V104I	G159D	P210I	T213R	A232V	Q236H	Q245R	T260A			
V68A	N76D	S103A	V104I	G159D	V205I	T213R	A232V	Q236H	Q245R	T260A			
V68A	N76D	S103A	V104I	G159D	P210I	A232V	Q236H	Q245R	T260A				
V68A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A					
N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A					
V68A	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R						
V68A	S103A	V104I	G159D	P210I	A232V	Q236H	Q245R						
V68A	S103A	V104I	G159D	A230V	A232V	Q236H	Q245R						
V68A	S103A	V104I	G159D	L126F	A232V	Q236H	Q245R						
V68A	S103A	V104I	G159D	V205I	A232V	Q236H	Q245R						
V68A	S103A	V104I	G159D	P210L	A232V	Q236H	Q245R						
S103A	V104I	G159D	A230V	Q236H	Q245R								
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	T260A						
S103A	V104I	G159D	A232V	Q236H	Q245R								
V68A	S103A	V104I	G159D	A174V	A232V	Q236H	Q245R	L257V					

V68A	S103A	V104I	G159D	A194S	A232V	Q236H	Q245R	L257V				
V68A	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	L257V				
S103A	V104I	G159D	A232V	Q236H	Q245R	L257V						
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A	N261W		
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V	N261W				
S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A					
S103A	V104I	G159D	P210I	A232V	Q236H	Q245R	N248D	N252K				
S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	L257V					
V68A	N76D	S103A	V104I	G159D	P210L	T213R	A232V	Q236H	Q245R	T260A		
Q12R	S103A	V104I	G159D	Y209W	T213R	A232V	Q236H	Q245R	T260A			
S103A	V104I	Y209W	A232V	Q236H	Q245R	L257V						
S103A	V104I	G159D	V205I	P210I	T213R	A232V	Q236H	Q245R	T260A			
S103A	V104I	G159D	V205I	Y209W	A232V	Q236H	Q245R	T260A				
V68A	S103A	V104I	G159D	V205I	Y209W	P210I	A232V	Q236H	Q245R			
S103A	V104I	G159D	V205I	Y209W	P210I	A232V	Q236H	Q245R	L257V			
S103A	V104I	G159D	V205I	Y209W	A232V	Q236H	Q245R	L257V				
V68A	S103A	V104I	G159D	V205I	Y209W	P210I	A232V	Q236H	Q245R	T260A		
S103A	V104I	G159D	V205I	Y209W	P210I	A232V	Q236H	Q245R				
S103A	V104I	G159D	Y209W	P210I	A232V	Q236H	Q245R					

S103A	V104I	G159D	V205I	P210I	A232V	Q236H	Q245R				
V68A	S103A	V104I	S128L	G159D	A232V	Q236H	Q245R				
A48V	S103A	V104I	G159D	A230V	Q236H	Q245R					
A48V	V68A	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R			
A48V	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		
A48V	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V	N261W		
G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K		
Q12R	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K	
S101G	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K	
A98L	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K	
G102A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
S103A	V104I	P131V	G159D	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	N184S	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	N184G	A232V	Q236H	Q245R	N248D	N252K			
S103A	V104I	G159D	A232V	Q236H	V244T	Q245R	N248D	N252K			
S103A	V104I	G159D	A232V	Q236H	V244A	Q245R	N248D	N252K			
N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K	S256R	
Q12R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K	
S101G	S103A	V104I	G159D	N185D	A232V	Q236H	Q245R	N248D	N252K		

S101G	S103A	V104I	G159D	Q206E	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	T213Q	A232V	Q236H	Q245R	N248D	N252K			
A98L	G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
A98L	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K		
A98L	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	N248D	N252K			
N62D	S103A	V104I	Q109R	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S103A	V104I	G159D	S212G	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S101G	S103A	V104I	G159D	S212G	T213R	A232V	Q236H	Q245R	N248D	N252K	
S103A	V104I	G159D	A232V	Q245R	N248D	N252K						
S103A	V104I	G159D	A230V	Q245R								
N62D	S103A	V104I	S130G	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
S101G	S103A	V104I	S130G	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	S128G	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	S128L	G159D	A232V	Q236H	Q245R	N248D	N252K			
N62D	S101G	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S103A	V104I	S128G	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S103A	V104I	S128L	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	T260A			

S101G	S103A	V104I	P131V	G159D	A232V	Q236H	Q245R	N248D	N252K			
A98V	S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S99G	S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	P210I	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	V205I	A232V	Q236H	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	A230V	Q236H	Q245R						
S101G	S103A	V104I	G159D	A194P	A232V	Q236H	Q245R	N248D	N252K			
N76D	S101G	S103A	V104I	G159D	A194P	A232V	Q236H	Q245R	N248D	N252K		
S101G	S103A	V104I	G159D	A230V	A232V	Q236H	Q245R	N248D	N252K			
N62D	S103A	V104I	G159D	N185D	Q206E	T213R	A232V	Q236H	Q245R	N248D	N252K	E271Q

Example 2

A large number of the protease variants produced in Example 1 were tested for performance in two types of detergent and wash conditions using a microswatch assay described in "An improved method of assaying for a preferred enzyme and/or preferred detergent composition", U.S. Serial No. 60/068,796.

Table 4 lists the variant proteases assayed and the results of testing in two different detergents. For column A, the detergent was 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column B, the detergent was 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 40°C.

Table 4

											A	B
N76D	S103A	V104I									1	1
S103A	V104I	A228T									0.56	1.11
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K				1.41	1.85
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			2.77	1.20
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R					2.26	1.67
V68A	S103A	V104I	N140D	G159D	A232V	Q236H	Q245R	N252K			2.96	1.42
N43S	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K			1.91	1.80
N43K	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R				2.05	1.78
N43D	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252K			2.00	1.34
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V				2.38	1.67
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D				2.83	0.53
V68A	S103A	V104I	G159D	A232V	Q236H	K237E	Q245R				2.87	0.20
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N252S				2.56	1.41
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V	R275H			3.97	0.47
V68A	S103A	V104I	G159D	T224A	A232V	Q236H	Q245R	L257V			3.35	1.28
G61E	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		3.77	0.09
N43D	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		3.50	0.47
V68A	S103A	V104I	G159D	S212P	A232V	Q236H	Q245R	N248D	N252K		2.81	1.46

N76D	A98E	S103A	V104I								1.56	0.28
V4E	N76D	S103A	V104I								1.22	0.33
N76D	N77D	S103A	V104I								1.13	0.36
A16T	N76D	S103A	V104I	N248D							1.22	0.43
A1E	N76D	S103A	V104I								1.12	0.32
N76D	S103A	V104I	N261D								1.54	0.33
N76D	S103A	V104I	S216C								1.04	0.13
N76D	N77D	S103A	V104I	A174V							1.09	0.35
T38S	N76D	S103A	V104I	K237Q							1.11	0.55
N76D	S103A	V104I	N183D								1.50	0.25
R19L	N76D	S103A	V104I								1.11	0.48
R19C	N76D	S103A	V104I								1.05	0.19
N76D	S103A	V104I	N184D								1.32	0.29
N76D	S103A	V104I	N252D								1.19	0.53
N76D	S103A	V104I	S259C								0.92	0.12
N76D	S103A	V104I	K251T								1.31	0.43
N76D	P86S	S103A	V104I								1.00	0.98
I72V	N76D	S103A	V104I	N185D							1.70	0.37
N76D	S103A	V104I	K237E	T274A							1.12	0.16
N76D	S103A	V104I	A228V								1.13	0.99

N76D	S103A	V104I	G159D								1.88	0.23
H17L	N76D	S103A	V104I	K237E							1.29	0.28
N76D	S103A	V104I	S130L								0.52	0.71
N76D	S103A	V104I	Q109R								0.23	1.26
N76D	S99R	S103A	V104I	N204T							0.21	0.87
N76D	S103A	V104I	D181N								0.24	1.07
Q12R	N76D	S103A	V104I								0.61	1.31
N76D	S103A	V104I	S212P	E271V							0.69	1.35
N76D	S103A	V104I	N252K	N261Y							0.37	1.02
N76D	S103A	V104I	S242T								0.98	0.92
N76D	S103A	V104I	E271Q								0.63	1.25
Q12R	N76D	S103A	V104I	S242T							0.49	1.32
N43S	N76D	S103A	V104I	N116K	N183I						0.39	1.10
N76D	S103A	V104I	G258R								0.34	1.17
N76D	S103A	V104I	E271G								0.57	1.25
N76D	S103A	V104I	Q182R	I198V							0.22	0.95
L21M	N76D	S103A	V104I	Q182R							0.24	0.98
N76D	S103A	V104I	M119I	Q137R							0.13	0.91
N76D	S103A	V104I	Q137R	N248S							0.16	1.02
A13T	N76D	S103A	V104I	Q206R							0.31	1.01

N76D	S103A	V104I	Q206R								0.33	1.02
N76D	S103A	V104I	S212P	G258R							0.38	1.06
T58S	N76D	S103A	V104I	E271G							0.84	1.26
N76D	S103A	V104I	Q206E	N261D							1.97	0.04
V4E	N76D	S103A	V104I	Q206E							1.51	0.05
N76D	N77D	S103A	V104I	Q206E							1.40	0.04
N76D	S103A	V104I	A158E								1.95	0.16
N76D	S103A	V104I	Q206E								2.41	0.88
N76D	N77D	S103A	V104I	A133T	N185D	K251T					1.34	0.03
N76D	S103A	V104I	Q206E	N261D							1.78	0.04
N76D	S103A	V104I	G159D	Q206E	V244A						2.16	0.04
V4E	N76D	S103A	V104I	S188E							1.91	0.04
V4E	N76D	S103A	V104I	A158E							2.06	0.04
N76D	S103A	V104I	Q206E	K251T							1.73	0.06
A48T	N76D	S103A	V104I	L111M	G159D						2.04	0.16
V68A	N76D	S103A	V104I	G159D	Q236H						3.20	0.09
L42V	N76D	S103A	V104I	G159D							1.83	0.17
Q12H	N62H	N76D	S103A	V104I	G159D						1.42	0.14
L42I	N76D	S103A	V104I	G159D							1.86	0.18
N76D	S103A	V104I	G146S	G159D							1.87	0.19

N76D	S103A	V104I	G159D	N238S							1.90	0.15
N76D	S103A	V104I	G159D	T224A							1.61	0.07
N76D	S103A	V104I	S212P	V268F	E271V						0.44	1.42
N76D	S87R	S103A	V104I	S212P	E271V						0.39	2.03
N76D	S103A	V104I	S212P	Q245L	E271V						0.62	1.79
N76D	S103A	V104I	Q109R	Q245R							0.11	1.78
N76D	S103A	V104I	Q109R	P210L							0.12	1.21
G20V	N62S	N76D	S103A	V104I							1.63	0.78
V68A	N76D	S103A	V104I	Q236H							2.37	0.44
V68A	N76D	S103A	V104I	G159D	Q236H	E271V					2.97	0.45
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R					3.00	0.61
V68A	N76D	S103A	V104I	G159D	L217I	Q236H	E271V				2.71	0.12
H17Q	V68A	N76D	S103A	V104I							2.46	0.38
V68A	N76D	S103A	V104I								2.46	0.61
V68A	N76D	S103A	V104I	G159D	Q236R						3.31	0.11
V68A	L75R	N76D	S103A	V104I	G159D	Q236H					3.06	0.14
V68A	N76D	S103A	V104I	A114V	V121I	G159D	Q236H	Q245R			3.11	0.40
Q12R	V68A	N76D	S103A	V104I	G159D	Q236H					3.12	0.34
V68A	N76D	S103A	V104I	G159D	Y209S	Q236H	T253K				3.18	0.03
V68A	N76D	S103A	V104I	N117K	G159D	N184S	Q236H				2.78	0.06

V68A	N76D	S103A	V104I	Q236H								2.49	0.57
V68A	N76D	S103A	V104I	G159D	Q236H	Q245L						3.37	0.03
V68A	N76D	S103A	V104I	N123S	G159D	Q236H	H249Y					3.11	0.03
V68A	N76D	S103A	V104I	G159D	Q236H	H249Q						3.15	0.04
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R	N261D					3.31	0.03
V68A	N76D	S103A	V104I	S141N	G159D	Q236H	Q245R	T255S				3.26	0.62
V68A	N76D	S103A	V104I	G159D	Q236H	Q245R	R247H					2.78	0.03
V68A	N76D	S103A	V104I	G159D	A174V	N204D	Q236H	Q245R				3.28	0.02
V68A	N76D	S103A	V104I	G159D	N204D	Q236H	Q245R					3.34	0.02
V68A	N76D	S103A	V104I	A133V	G159D	N218D	Q236H	Q245R				3.28	0.03
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R					2.91	0.58
V68A	N76D	S103A	V104I	G159D	A194I	V203A	Q236H	Q245R				2.86	0.13
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A			1.30	1.73
T22K	V68A	N76D	S103A	V104I								1.83	1.13
V68A	N76D	S103A	V104I	G159D	P210R	A232V	Q236H	Q245R				1.28	1.54
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V				3.72	0.8
N76D	S103A	V104I	A232V	Q236H	Q245R	L257V						0.6	1.5
N76D	S103A	V104I	L257V	R275H								1.91	0.15
N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V					1.92	1.09
V68A	N76D	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R				3.57	0.99

V68A	N76D	S103A	V104I	G159D	G211R	A232V	Q236H	Q245R			1.74	1.76
V68A	N76D	S103A	V104I	G159D	G211V	A232V	Q236H	Q245R			3.15	1.06
Q12R	V68A	N76D	S103A	V104I	G159D	Y214L	A232V	Q236H	Q245R		2.33	1.92
V68A	N76D	S103A	V104I	G159D	A215R	A232V	Q236H	Q245R			1.67	1.45
Q12R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R			2.16	1.72
G20R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	S259G		2.77	1.59
V68A	N76D	S87R	S103A	V104I	G159D	A232V	Q236H	Q245R	T260V		2.62	1.49
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N261G			2.92	0.68
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N261W			2.17	1.37
N76D	S103A	V104I	A232V	Q236H	S242P	Q245R					0.48	1.2
V68A	N76D	S103A	V104I	G159D	P210L	A232V	Q236H	Q245R			2.92	0.76
Q12R	A48V	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R		2.09	1.86
N76D	S103A	V104I	A232V	Q236H	Q245R						0.51	1.44
N76D	S103A	V104I	G159D	Y192F	A232V	Q236H	Q245R				1.60	1.14
N76D	S103A	V104I	V147I	G159D	A232V	Q236H	Q245R	N248S	K251R		1.35	1.29
Q12R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	A272S		1.92	1.81
V68A	N76D	S103A	V104I	G159D	N183K	Q206L	A232V	Q236H	Q245R		1.17	1.53
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	S256R			2.01	1.72
V68A	N76D	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R			2.09	1.62
K27R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R			3.00	1.08

V68A	N76D	S103A	V104I	N116T	G159D	R170S	N185S	A232V	Q236H	Q245R	ND	ND
N76D	S103A	V104I	M222S	Q245R							1.01	1.23
Q12R	N76D	S103A	V104I	M222S	H249R						0.57	1.65
N76D	S103A	V104I	N173R	M222S							0.86	0.46
N76D	S103A	V104I	M222S	Y263F							1.24	0.77
L21M	N76D	S103A	V104I	M222S	K237R	Y263F					1.18	0.76
N76D	S103A	V104I	Q109R	M222S							0.52	1.16
N76D	S103A	V104I	Q109R	M222S	E271D						0.56	1.12
G61R	N76D	S103A	V104I	M222S							0.43	0.96
N76D	S103A	V104I	Q137R	M222S							0.42	1.25
N76D	S103A	V104I	M222S	H249R							1.15	1.01
Q12R	N76D	S103A	V104I	M222S	Q245R						0.53	1.46
N76D	S103A	V104I	A232V	Q245R							0.69	1.56
Q12R	N76D	S103A	I104T	M222S	V244I	Q245R					0.66	1.74
Q12R	N76D	S103A	V104I	M222S	P210T	Q245R					0.52	1.56
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R					0.70	1.61
Q12R	N76D	S103A	I104T	S130T	A215V	M222S	Q245R				0.79	1.85
Q12R	N76D	S103A	I104T	S130T	M222S	V227A	Q245R	L262S			0.78	1.56
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R	N261D				1.25	1.30
N76D	S103A	I104T	S130T	M222S	Q245R						1.29	1.30

Q12R	S57P	N76D	S103A	I104T	S130T	M222S	Q245R	K251Q			1.44	0.16
Q12R	N76D	S103A	I104T	S130T	R170S	N185D	M222S	N243D	Q245R		2.01	0.04
Q12R	N76D	S103A	I104T	S130T	M222S	Q245R	V268A				0.77	1.60
Q12R	N76D	S103A	I104T	S130T	M222S	P210S	Q245R				0.73	1.66
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R				2.09	0.86

Example 3

Table 5 lists the variant proteases assayed from Example 1 and the results of testing in four different detergents. The same performance tests as in Example 2 were done on the noted variant proteases with the following detergents. For column A, the detergent was 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column B, the detergent was 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 40°C. For column C, 3.5g/l HSP1 detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 8 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column D, 1.5 ml/l Tide KT detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca^{2+}/Mg^{2+} hardness, and 0.3 ppm enzyme was used in each well at 20°C.

Table 5

													A	B	C	D
N76D	S103A	V104I											1	1	1	1
S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K						1.44	1.41	1.39	1.26
V68A	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	N248D	N252K				2.34	1.49	1.65	2.35
V68A	S103A	V104I	Q109R	G159D	A232V	Q236H	Q245R	N248D	N252K				1.05	1.41	1.20	1.19
G20R	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				1.81	1.72	1.66	1.31
V68A	S103A	V104I	G159D	Y209F	A232V	Q236H	Q245R	N248D	N252K				2.19	1.38	1.60	2.02
V68A	S103A	V104I	G159D	N185D	A232V	Q236H	Q245R	N248D	N252K				2.91	0.91	1.48	2.70
V68A	S103A	V104I	G159D	P210R	A232V	Q236H	Q245R	N248D	N252K				0.93	1.39	1.23	0.80
V68A	S103A	V104I	G159D	N185D	P210L	A232V	Q236H	Q245R	N248D	N252K			2.67	0.86	1.41	2.88
V68A	S103A	V104I	G159D	P210L	A232V	Q236H	Q245R	N248D	N252K				2.22	1.43	1.55	1.78
V68A	S103A	V104I	G159D	S212C	A232V	Q236H	Q245R	N248D	N252K				2.30	1.43	1.63	2.07
V68A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K				2.31	1.47	1.62	2.01
V68A	S103A	V104I	G159D	S212E	A232V	Q236H	Q245R	N248D	N252K				2.63	0.56	1.36	2.66
V68A	S103A	V104I	G159D	T213E	A232V	Q236H	Q245R	N248D	N252K				2.75	0.50	1.27	2.78
V68A	S103A	V104I	T213S	A232V	Q236H	Q245R	N248D	N252K					1.11	1.38	1.31	0.75
V68A	A103V	V104I	G159D	T213E	A232V	Q236H	Q245R	N248D	N252K				2.27	0.15	1.12	2.01
V68A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K				1.37	1.42	1.37	1.06
V68A	S103A	V104I	G159D	A215V	A232V	Q236H	Q245R	N248D	N252K				2.14	1.40	1.53	1.54

V68A	S103A	V104I	G159D	A215R	A232V	Q236H	Q245R	N248D	N252K				1.22	1.58	1.47	1.20
V68A	S103A	V104I	G159D	S216T	A232V	Q236H	Q245R	N248D	N252K				2.12	1.36	1.56	1.56
V68A	S103A	V104I	G159D	S216V	A232V	Q236H	Q245R	N248D	N252K				1.88	1.36	1.47	1.87
V68A	S103A	V104I	G159D	S216C	A232V	Q236H	Q245R	N248D	N252K				2.24	0.33	1.07	2.89
V68A	S103A	V104I	G159D	N173D	A232V	Q236H	Q245R	N248D	N252K				2.43	0.46	1.29	2.42
V68A	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R	N248D	N252K				0.98	1.46	1.24	0.95
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252F					2.52	1.00	1.42	2.42
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252L					2.05	1.13	1.30	1.85
P55S	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252F				2.61	0.91	1.43	3.22
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	T255V				2.18	1.36	1.58	1.72
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256N				2.14	1.46	1.59	1.65
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256E				2.46	0.77	1.33	2.58
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	S256R				1.31	1.52	1.46	0.94
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	T260R				1.21	1.41	1.31	1.05
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	L257R				1.51	1.41	0.85	1.18
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	G258D				2.56	0.59	1.30	2.64
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261R				1.02	1.47	1.37	0.84
V68A	S103A	V104I	A232V	Q236H	Q245R	N248D	N252K						1.04	1.50	1.32	0.73
V68A	S103A	V104I	G159D	A232V	Q236H	Q245V	N248D	N252K					2.60	0.93	1.41	2.67
V68A	S103A	V104I	G159D	A228V	A232V	Q236H	Q245R	N248D	N252K				2.31	1.38	1.53	1.57
G61E	V68A	S103A	V104I	S130A	G159D	A232V	Q236H	Q245R	N248D	N252K			2.83	0.25	1.33	2.44

G61E	S103A	V104I	A133V	G159D	A232V	Q236H	Q245R	N248D	N252K				2.10	0.97	1.36	2.29
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248G	N252K					1.37	1.54	0.89	1.27
V68A	S103A	V104I	G159D	N218S	A232V	Q236H	Q245R	N248D	N252K				2.30	1.50	1.62	1.56
G20R	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				1.72	1.72	1.67	1.15
V68A	N76D	E89D	S103A	V104I	G159D	P210L	T213R	A232V	Q236H	Q245R	T260A		1.32	1.30	1.11	1.28
V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				2.50	0.83	1.43	2.25
G61E	V68A	S103A	V104I	G159D	S160V	A232V	Q236H	Q245R	N248D	N252K			4.20	0.07	ND	1.28
S3L	G61E	V68A	N76D	S103A	V104I	A232V	Q236H	Q245R	N248D	N252K			3.47	0.60	ND	1.45
G61E	V68A	S103A	V104I	G159D	Y167F	A232V	Q236H	Q245R	N248D	N252K			4.32	0.79	ND	1.55
G97E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					3.14	0.41	ND	1.40
A98D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					2.71	0.68	ND	1.72
S99E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					2.97	0.68	ND	1.71
S101E	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					3.50	0.27	ND	1.90
S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					2.24	1.80	ND	1.33
G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					3.35	1.33	ND	1.69
S103A	V104I	S106E	G159D	A232V	Q236H	Q245R	N248D	N252K					4.88	0.55	ND	2.71
S103A	V104I	Q109E	G159D	A232V	Q236H	Q245R	N248D	N252K					4.22	1.05	ND	2.40
S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K	N261R					5.45	2.19	ND	2.58
S103A	V104I	Q109R	G159D	A232V	Q236H	Q245R	N248D	N252K					3.76	2.16	ND	1.82
N62D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					7.42	0.13	ND	2.46
S103A	V104I	G159D	N184D	A232V	Q236H	Q245R	N248D	N252K					5.43	1.36	ND	2.84

S103A	V104I	G159D	S166D	A232V	Q236H	Q245R	N248D	N252K					5.12	1.21	ND	3.97
S103A	V104I	G159D	L217E	A232V	Q236H	Q245R	N248D	N252K					6.38	0.95	ND	3.09
G20R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K			3.17	2.83	ND	2.60
N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K				4.38	1.92	ND	2.54
S103A	V104I	G159D	Q206R	L217E	A232V	Q236H	Q245R	N248D	N252K				3.05	2.61	ND	1.10
N62D	S103A	V104I	G159D	Q206R	A232V	Q236H	Q245R	N248D	N252K				4.09	2.46	ND	2.55
S103A	V104I	G159D	N184G	A232V	Q236H	Q245R	N248D	N252K					2.32	2.08	ND	2.40
S103A	V104I	G159D	A232V	Q236H	V244T	Q245R	N248D	N252K					2.34	2.04	ND	1.86
S103A	V104I	G159D	A232V	Q236H	V244A	Q245R	N248D	N252K					2.24	2.11	ND	1.95
K27N	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					2.81	1.56	ND	2.47
T38G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K					2.30	2.09	ND	1.82
N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K	S256R			2.63	2.66	ND	1.44
Q12R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K			2.01	2.78	ND	1.99
N62D	S103A	V104I	G159D	N185D	Q206E	T213R	A232V	Q236H	Q245R	N248D	N252K	E271Q	7.74	0.94	ND	5.39
S101G	S103A	V104I	G159D	N185D	A232V	Q236H	Q245R	N248D	N252K				5.14	1.41	ND	1.92
S101G	S103A	V104I	G159D	Q206E	A232V	Q236H	Q245R	N248D	N252K				4.97	0.57	ND	1.36
S101G	S103A	V104I	G159D	T213Q	A232V	Q236H	Q245R	N248D	N252K				2.41	1.86	ND	1.01
A98L	G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				4.42	0.50	ND	2.88
S101G	G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				5.86	1.20	ND	3.84
G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K				5.87	2.10	ND	3.19
Q12R	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K			2.98	2.67	ND	2.17

A98L	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K		4.02	0.41	ND	2.25
S101G	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K		6.63	2.07	ND	2.08
G102A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K			2.03	2.48	ND	2.25
N62D	S103A	V104I	Q109R	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		2.96	2.76	ND	2.34
S103A	V104I	G159D	A232V	Q245R	N248D	N252K						2.74	2.10	ND	1.86
S103A	V104I	G159D	A230V	Q245R								2.11	2.35	ND	1.49
N62D	S103A	V104I	S130G	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		3.42	0.71	ND	2.58
S101G	S103A	V104I	S130G	G159D	A232V	Q236H	Q245R	N248D	N252K			2.59	1.32	ND	1.61
S101G	S103A	V104I	S128G	G159D	A232V	Q236H	Q245R	N248D	N252K			1.30	1.23	ND	9.0
S101G	S103A	V104I	S128L	G159D	A232V	Q236H	Q245R	N248D	N252K			2.94	0.71	ND	1.08
N62D	S101G	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		3.17	0.83	ND	2.35
N62D	S103A	V104I	S128G	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		2.15	1.38	ND	1.77
N62D	S103A	V104I	S128L	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		3.07	0.07	ND	1.45
S101G	S103A	V104I	P131V	G159D	A232V	Q236H	Q245R	N248D	N252K			2.26	1.16	ND	3.05
A98V	S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			1.82	1.34	ND	1.08
S99G	S101G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			2.16	1.47	ND	1.20
S101G	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K			1.79	1.38	ND	1.01
S101G	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	N248D	N252K			1.15	1.18	ND	8.7
S101G	S103A	V104I	G159D	P210I	A232V	Q236H	Q245R	N248D	N252K			1.47	1.23	ND	1.03
S101G	S103A	V104I	G159D	V205I	A232V	Q236H	Q245R	N248D	N252K			1.90	1.38	ND	1.05
S101G	S103A	V104I	G159D	A230V	Q236H	Q245R						1.55	1.51	ND	1.23

S101G	S103A	V104I	G159D	A194P	A232V	Q236H	Q245R	N248D	N252K				1.96	1.30	ND	1.10
N76D	S101G	S103A	V104I	G159D	A194P	A232V	Q236H	Q245R	N248D	N252K			2.49	0.80	ND	1.25

WHAT IS CLAIMED:

1. A protease variant comprising substituting an amino acid at a residue position corresponding to position 103 of *Bacillus amyloliquefaciens* subtilisin and substituting one or more amino acids at residue positions selected from the group consisting of residue positions corresponding to positions 1, 3, 4, 8, 10, 12, 13, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274 and 275 of *Bacillus amyloliquefaciens* subtilisin; wherein when a substitution at a position corresponding to residue position 103 is combined with a substitution at a position corresponding to residue position 76, there is also a substitution at one or more residue positions other than residue positions corresponding to positions 27, 99, 101, 104, 107, 109, 123, 128, 166, 204, 206, 210, 216, 217, 218, 222, 260, 265, or 274 of *Bacillus amyloliquefaciens* subtilisin.

2. The protease variant according to claim 1 which is derived from a *Bacillus* subtilisin.

3. The protease variant according to claim 2 which is derived from *Bacillus lentus* subtilisin.

4. A DNA encoding a protease variant of claim 1.

5. An expression vector encoding the DNA of claim 4.

6. A host cell transformed with the expression vector of claim 5.

7. A cleaning composition comprising the protease variant of claim 1.

8. An animal feed comprising the protease variant of claim 1.

9. A composition for treating a textile comprising the protease variant of claim 1.

10. The protease variant according to claim 1 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 1 of *Bacillus amyloliquefaciens* subtilisin.

11. The protease variant according to claim 10 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 3 of *Bacillus amyloliquefaciens* subtilisin.

12. The protease variant according to claim 10 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 2 of *Bacillus amyloliquefaciens* subtilisin.

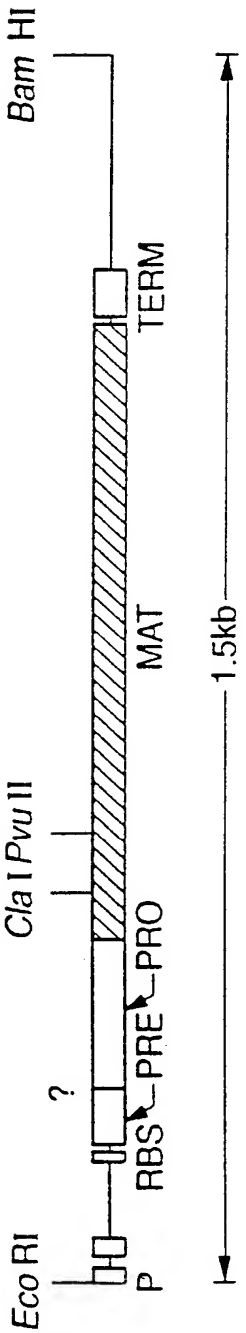

FIG._1A

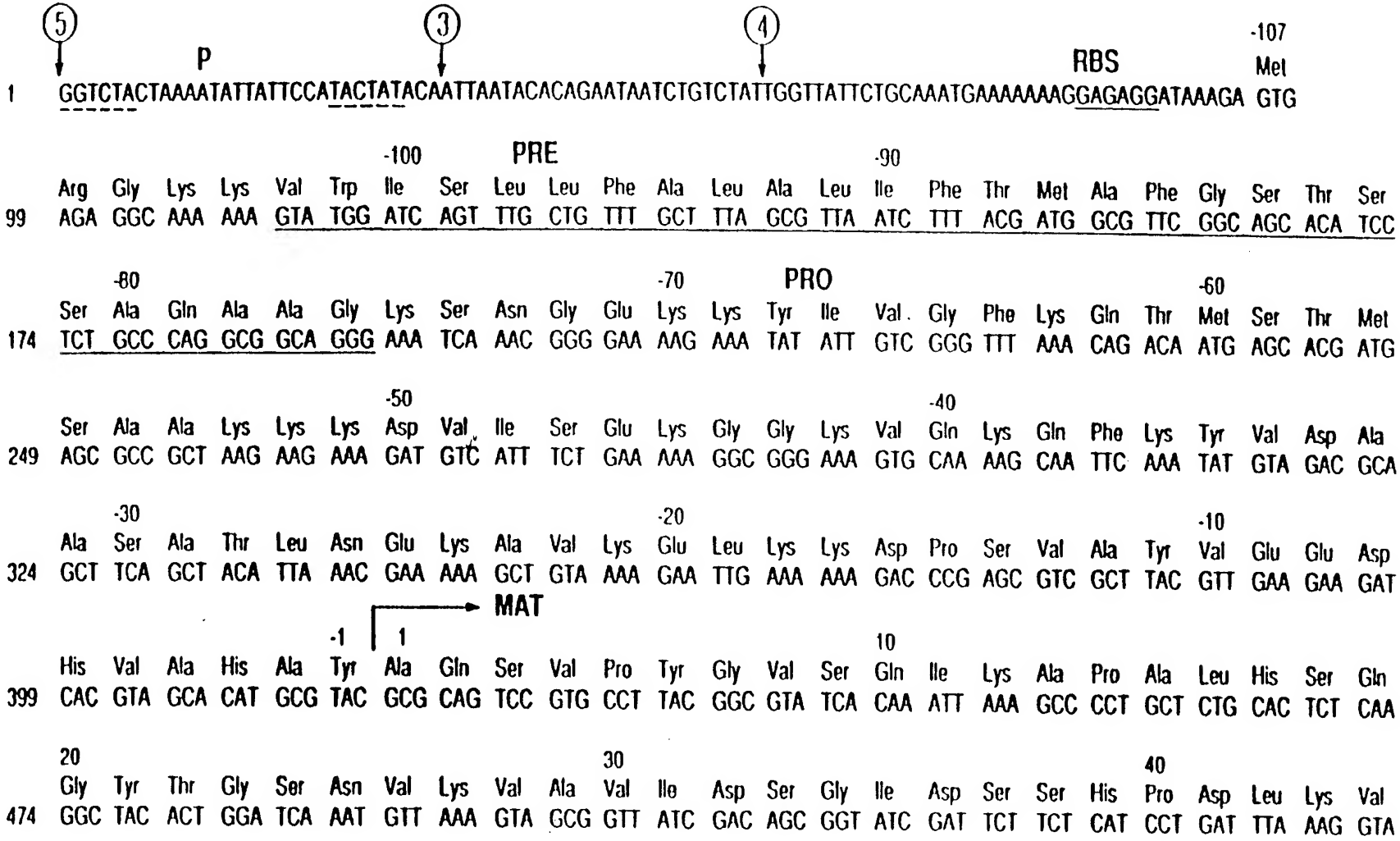
5
3
4
P
RBS
-107
Met
1 GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGA GTG
-100
PRE
-90
Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
99 AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC
-80
-70
PRO
-60
Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
174 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG
-50
-40
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala
249 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA
-30
-20
-10
Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp
324 GCT TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT
MAT
-1
1
10
His Val Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln
399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA
20
30
40
Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA

FIG._1B - 1

					50						Pro	Asn			60	Asp								
	Ala	Gly	Gly	Ala	Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His	Gly	Thr	His	Val	Ala
549	GCA	GGC	GGA	GCC	AGC	ATG	GTT	CCT	TCT	GAA	ACA	AAT	CCT	TTC	CAA	GAC	AAC	AAC	TCT	CAC	GGA	ACT	CAC	GTT	GCC
	70										80								Ser	Ala	90				
	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys
624	GGC	ACA	GTT	GCG	GCT	CTT	AAT	AAC	TCA	ATC	GGT	GTA	TTA	GGC	GTT	GCG	CCA	AGC	GCA	TCA	CTT	TAC	GCT	GTA	AAA
				Asp	Ala	100										110									
	Val	Leu	Gly	Ala	Asp	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	Trp	Ala	Ile	Ala	Asn	Asn	Met
699	GTT	CTC	GGT	GCT	GAC	GGT	TCC	GGC	CAA	TAC	AGC	TGG	ATC	ATT	AAC	GGA	ATC	GAG	TGG	GCG	ATC	GCA	AAC	AAT	ATG
	120										130										140				
	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Pro	Ser	Gly	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp	Lys	Ala	Val	Ala
774	GAC	GTT	ATT	AAC	ATG	AGC	CTC	GGC	GGA	CCT	TCT	GGT	TCT	GCT	GCT	TTA	AAA	GCG	GCA	GTT	GAT	AAA	GCC	GTT	GCA
						150								Ser	Thr	160									
	Ser	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Thr	Ser	Gly	Ser	Ser	Ser	Thr	Val	Gly	Tyr	Pro	Gly
849	TCC	GGC	GTC	GTA	GTC	GTT	GCG	GCA	GCC	GGT	AAC	GAA	GGC	ACT	TCC	GGC	AGC	TCA	AGC	ACA	GTG	GGC	TAC	CCT	GGT
	170										180										190				
	Lys	Tyr	Pro	Ser	Val	Ile	Ala	Val	Gly	Ala	Val	Asp	Ser	Ser	Asn	Gln	Arg	Ala	Ser	Phe	Ser	Ser	Val	Gly	Pro
924	AAA	TAC	CCT	TCT	GTC	ATT	GCA	GTA	GGC	GCT	GTT	GAC	AGC	AGC	AAC	CAA	AGA	GCA	TCT	TTC	TCA	AGC	GTA	GGA	CCT
						200										210									
	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	Leu	Pro	Gly	Asn	Lys	Tyr	Gly	Ala	Tyr	Asn	Gly
999	GAG	CTT	GAT	GTC	ATG	GCA	CCT	GGC	GTA	TCT	ATC	CAA	AGC	ACG	CTT	CCT	GGA	AAC	AAA	TAC	GGG	GCG	TAC	AAC	GGT
	220										230										240				
	Thr	Ser	Met	Ala	Ser	Pro	His	Val	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	Ser	Lys	His	Pro	Asn	Trp	Thr	Asn	Thr
1074	ACG	TCA	ATG	GCA	TCT	CCG	CAC	GTT	GCC	GGA	GCG	GCT	GCT	TTG	ATT	CTT	TCT	AAG	CAC	CCG	AAC	TGG	ACA	AAC	ACT

FIG._1B - 2

```
                                  250  Gln                                                  260
     Gln  Val  Arg  Ser  Ser  Leu  Glu  Asn  Thr  Thr  Thr  Lys  Leu  Gly  Asp  Ser  Phe  Tyr  Tyr  Gly  Lys  Gly  Leu  Ile  Asn
1149 CAA  GTC  CGC  AGC  AGT  TTA  GAA  AAC  ACC  ACT  ACA  AAA  CTT  GGT  GAT  TCT  TTC  TAC  TAT  GGA  AAA  GGG  CTG  ATC  AAC

     270                       275
     Val  Gln  Ala  Ala  Ala  Gln  OC                TERM
1224 GTA  CAG  GCG  GCA  GCT  CAG  TAA  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
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FIG._1B - 3

FIG._1B

FIG._1B - 1

FIG._1B - 2

FIG._1B - 3

CONSERVED RESIDUES IN SUBTILISINS FROM *BACILLUS AMYLOLIQUEFACIENS*

```
1                   10                  20
A Q S V P . G . . . . . A P A . H . . G

21                  30                  40
. T G S . V K V A V . D . G . . . . H P

41                  50                  60
D L . . . G G A S . V P . . . . . Q D

61                  70                  80
. N . H G T H V A G T . A A L N N S I G

81                  90                  100
V L G V A P S A . L Y A V K V L G A . G

101                 110                 120
S G . . S . L . . G . E W A . N . . . .

121                 130                 140
V . N . S L G . P S . S . . . . . A . .

141                 150                 160
. . . . . G V . V V A A . G N . G . . .

161                 170                 180
. . . . . Y P . . Y . . . . A V G A .

181                 190                 200
D . . N . . A S F S . . G . . L D . . A

201                 210                 220
P G V . . Q S T . P G . . Y . . . N G T

221                 230                 240
S M A . P H V A G A A A L . . . K . . .

241                 250                 260
W . . . Q . R . . L . N T . . . L G . .

261                 270
. . Y G . G L . N . . A A . .
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FIG._2

COMPARISON OF SUBTILISIN SEQUENCES FROM:

B.amyloliquefaciens
B.subtilis
B.licheniformis
B.lentus

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01                  10                  20                  30
A Q S V P Y G V S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q S V P Y G I S Q I K A P A L H S Q G Y T G S N V K V A V I D S G I D S S H P
A Q T V P Y G I P L I K A D K V Q A Q G F K G A N V K V A V L D T G I Q A S H P
A Q S V P W G I S R V Q A P A A H N R G L T G S G V K V A V L D T G I S T * H P

41                  50                  60                  70
D L K V A G G A S M V P S E T N P F Q D N N S H G T H V A G T V A A L N N S I G
D L N V R G G A S F V P S E T N P Y Q D G S S H G T H V A G T I A A L N N S I G
D L N V V G G A S F V A G E A Y N * T D G N G H G T H V A G T V A A L D N T T G
D L N I R G G A S F V P G E * P S T Q D G N G H G T H V A G T I A A L N N S I G

81                  90                  100                 110
V L G V A P S A S L Y A V K V L G A D G S G Q Y S W I I N G I E W A I A N N M D
V L G V S P S A S L Y A V K V L D S T G S G Q Y S W I I N G I E W A I S N N M D
V L G V A P S V S L Y A V K V L N S S G S G S Y S G I V S G I E W A T T N G M D
V L G V A P S A E L Y A V K V L G A S G S G S V S S I A Q G L E W A G N N G M H

121                 130                 140                 150
V I N M S L G G P S G S A A L K A A V D K A V A S G V V V V A A A G N E G T S G
V I N M S L G G P T G S T A L K T V V D K A V S S G I V V A A A A G N E G S S G
V I N M S L G G A S G S T A M K Q A V D N A Y A R G V V V V A A A G N S G N S G
V A N L S L G S P S P S A T L E Q A V N S A T S R G V L V V A A S G N S G A G S
```

FIG._3A

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161                 170                 180                 190
S S S T V G Y P G K Y P S V I A V G A V D S S N Q R A S F S S V G P E L D V M A
S T S T V G Y P A K Y P S T I A V G A V N S S N Q R A S F S S A G S E L D V M A
S T N T I G Y P A K Y D S V I A V G A V D S N S N R A S F S S V G A E L E V M A
* * * * I S Y P A R Y A N A M A V G A T D Q N N N R A S F S Q Y G A G L D I V A

201                 210                 220                 230
P G V S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S K H P N
P G V S I Q S T L P G G T Y G A Y N G T S M A T P H V A G A A A L I L S K H P T
P G A G V Y S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S K H P N
P G V N V Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S

241                 250                 260                 270
W T N T Q V R S S L E N T T T K L G D S F Y Y G K G L I N V Q A A A Q
W T N A Q V R D R L E S T A T Y L G N S F Y Y G K G L I N V Q A A A Q
L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A A Q
W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R
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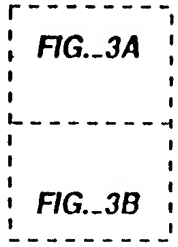
FIG._3B

FIG._3



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(57) Abstract

Novel protease variants derived from the DNA sequences of naturally–occurring or recombinant non–human proteases are disclosed. The variant proteases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding the naturally–occurring or recombinant protease to generate the substitution of a plurality of amino acid residues in the amino acid sequence of a precursor protease. Such variant proteases have properties which are different from those of the precursor protease, such as altered wash performance. The substituted amino acid residue corresponds to position 103 in combination with one or more of the following substitutions at residue positions corresponding to positions 1, 3, 4, 8, 10, 12, 13, 15, 16, 17, 18, 19, 20, 21, 22, 24, 27, 33, 37, 38, 42, 43, 48, 55, 57, 58, 61, 62, 68, 72, 75, 76, 77, 78, 79, 86, 87, 89, 97, 98, 99, 101, 102, 104, 106, 107, 109, 111, 114, 116, 117, 119, 121, 123, 126, 128, 130, 131, 133, 134, 137, 140, 141, 142, 146, 147, 158, 159, 160, 166, 167, 170, 173, 174, 177, 181, 182, 183, 184, 185, 188, 192, 194, 198, 203, 204, 205, 206, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 222, 224, 227, 228, 230, 232, 236, 237, 238, 240, 242, 243, 244, 245, 246, 247, 248, 249, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 265, 268, 269, 270, 271, 272, 274, and 275 of *Bacillus amyloliquefaciens* subtilisin, wherein when a substitution at a position corresponding to residue position 103 is combined with a substitution at a position corresponding to residue position 76, there is also a substitution at one or more residue positions other than residue positions corresponding to positions 27, 99, 101, 104, 107, 109, 123, 128, 166, 204, 206, 210, 216, 217, 218, 222, 260, 265 or 274 of *Bacillus amyloliquefaciens* subtilisin.

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INTERNATIONAL SEARCH REPORT

International Application No
P../US 98/22572

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N9/54 C11D3/386 A23K1/165

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 30011 A (PROCTER & GAMBLE) 9 November 1995 (1995-11-09)	1
Y	tables 8-10,33-35	1-11

X	WO 95 30010 A (PROCTER & GAMBLE) 9 November 1995 (1995-11-09)	1
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X	WO 91 00345 A (NOVONORDISK AS) 10 January 1991 (1991-01-10)	1-10
Y	claims 1,2,4	1-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 April 1999	19.07.99

Name and mailing address of the ISA	Authorized officer
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1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22572

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 10615 A (GENENCOR INT) 20 April 1995 (1995-04-20) the whole document ---	10,11
Y	US 5 316 935 A (ARNOLD FRANCES H ET AL) 31 May 1994 (1994-05-31) the whole document especially example XIII ---	1-9
Y	US 5 543 302 A (BOGUSLAWSKI GEORGE ET AL) 6 August 1996 (1996-08-06) claim 5 -----	1-9

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INTERNATIONAL SEARCH REPORT

ırnational application No.
PCT/US 98/ 22572

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
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2. ☐ Claims Nos.:
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because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-11 partially (invention 1. on continuation-sheet)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Invention 1: Claims 1-11 partially

Subtilisin variants with an amino acid substitution at position 103 plus at least one amino acid substitution at position 1, DNA encoding these variants and their uses.

Inventions 2-136: Claims 1-11 partially 12 completely

Invention 2 being subtilisin variants with an amino acid substitution at position 103 plus at least one at position 3, but not at position 1, DNA encoding these variants and their uses; invention 3 being subtilisin variants with an amino acid substitution at position 103 plus at least one at position 4, but not at position 1 or 3, DNA encoding these variants and their uses etc.. Invention 136 being subtilisin variants with an amino acid substitution at position 103 plus one at position 275, DNA encoding these variants and their uses

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/US 98/22572

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